CHARACTERIZATION OF THE ROOTING COFACTORS EXTRACTED FROM HEDERA *HELIX L. AND HIBISCUS ROSA-SINENSIS L.*

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Following the discovery of four root promoting substances in the easy-to-root juvenile form of Hedera helix. L. and the red flowering Hibiscus rosa-sinensis. L. (2), a procedure was developed to isolate and purify the substances in larger quantities. The rooting cofactors will be referred to by numbers, ie., cofactor 1, etc. The basis of the numbers was the relative position of the cofactors on a chromatogram developed in isopropanol and water (8:2 v/v) solvent system. The R_f values of the cofactors in this solvent system were as follows: cofactor 1, 0.0-0.13; cofactor 2, 0.33-0.53; cofactor 3, 0.60-0.73; and cofactor 4, 0.80-0.93.

Materials and Methods

Figure 1 shows the procedure which was developed to separate the cofactors in larger quantities than was possible with paper chromatography. A methanolic or ethanolic extract of lyophilized tissue from juvenile Hedera or red Hibiscus was evaporated to dryness. The dried residue was taken up in a mixture of 25cc of chloroform and 25cc of water and was transferred to a separatory funnel. The upper, water, layer contained cofactors 1, 2 and 3. The lower, chloroform, layer contained cofactor 4 and the chlorophyll pigments.

Cofactor 3 was separated from cofactors 1 and 2 by adjusting the pH value of the aqueous layer to 3.5 and then partitioning with ether. The upper, ether, layer contained cofactor 3. Up to the present cofactors 1 and 2 have been separated only by paper chromatography.

After partitioning cofactor 4 was purified by one of two methods. The chloroform fraction was concentrated and applied to a paper chromatogram, and a solvent system consisting of methanol and water (7:3 v/v) was used for development. The chlorophylls stayed at the origin and cofactor 4 moved to an R_f value of 0.73. An alternative method the chloroform fraction was passed through a column of Darco G-60 charcoal mixed with celite (Johns-Manville filtering aid). The two parts charcoal and one part celite on a weight basis were used. Most of cofactor 4 in the extract passes through the column in the clarified chloroform. Recovery was improved by flushing the column with a few portions of fresh chloroform after the initial extract had passed through the column. The clarified chloroform containing cofactor 4 was concentrated under vacuum to a few cc. The small amount of extract was then transferred to approximately 0.25 gm of sillica gel and evaporated. The sillica gel containing the extract was then placed on top of a 1 X 12 cm column of silica gel and the column was developed with a 7:3 (v/v) mixture of methanol and water. When the eluate was

collected in 3 cc fractions, cofactor 4 was eluted in the third fraction.

Chemical and Physical Characteristics of Cofactor 4

Cofactor 4 is an oily, yellow liquid at room temperature. It is only slightly soluble in water, but is soluble enough to be physiologically active. An aqueous solution of cofactor 4 is slightly acid. The solubility of cofactor 4 in other solvents is presented in Table 1.

Cofactor 4 has an outstanding deep blue fluorescence in ultraviolet light (254 mu) but does not have a characteristic ultraviolet absorption curve. Only end absorption was obtained. Cofactor 4 was subjected to a number of reagents. The procedure was to spot a small amount of the cofactor on Whatman No. 3 mm paper and spray the area with a reagent. The reagents and the results are presented in Table 2.

Biological Activity of Compounds Structurally Related to Cofactor 4

From the solubility characteristics, the fact that cofactor 4 was slightly acid in aqueous solution, the positive reactions with diazotized sulfanilic acid, and the coupling reaction with B-napthol, there was an indication that cofactor 4 was an aminophenol. Ortho aminophenol was tested in the mung bean rooting test and did have activity. The question was then asked whether the amino group or the dydroxyl group affected the promotional activity. To answer this question o-amino phenol was compared with aniline, phenol, anthranilic acid, and catechol. All compounds were supplied to the cuttings in combination with IAA at 5×10^{-6}M. A range of concentrations was used for each compound although only one concentration is shown in Figure 2. The average number of roots in the 5×10^{-6}IAA column was used as the control.

As can be seen in Figure 2 aniline did not have any activity and may have been slightly inhibitory. Phenol did promote rooting slightly, and anthranillic acid had no activity. It was concluded that the amino group on the benzene ring did not affect biological activity insofar as promoting root initiation was concerned. However, the hydroxyl group did affect activity, particularly when two hydroxyl groups were present as in catechol.

Next the activity of other di- and trihydric phenols was determined. As can be seen in Figure 3 the position of the hydroxyl group determines whether or not a phenolic compound will stimulate root initiation. Resorcinol, hydroxyl groups in the meta position and hydroqulnone, hydroxyl groups in the para position, were not active. Pyrogallol having three hydroxyl groups in a vicinal arrangement was highly active. Phloroglucinol, with a symmetrical arrangement of the hydroxyl groups, was not active.

Caffeic acid was included in the test because it was found in the extracts of Hedera and Hibiscus and from a structural standpoint seemed to meet the requirements for activity. However, even though there were two hydroxyl groups in the ortho position, caffeic acid was not active. This result suggested that perhaps the para position must be open in order for a compound to be biologically active in the rooting test.

To investigate this hypothesis several more compounds were tested. As can be seen in Figure 4, the para position must be free. Gallic acid with a carboxy group in the para position eliminated the activity of pyrogallol, and a hydroxyl group in 1,2,4- Benzenetriol eliminated its activity. Also, chlorogenic acid and caffeic acid were not active.

Therefore, structural requirements for a phenolic compound to stimulate root initiation are that at least 2 hydroxyl groups be present in an ortho relationship and that the para position must be free. The mode of action of catechol and pyrogallol are presently under study. As can be seen in Table 3, although catechol will stimulate root initiation alone, it reacts synergistically with IAA. Since the mung bean is a rich source of phenolase enzymes and since catechol is readily oxidized to a quinone it may be possible that oxidation of the ortho-dihydroxy phenol is one of the first steps leading to root initiation as suggested by Bouillenne and Bouillenne-Walrand (1). Furthermore, Leopold and Plummer suggest that IAA forms addition products with quinones produced by the action of phenolases upon catechol and other phenois (3). In order to determine if oxidation of catechol is one of the first steps, 2 phenolase inhibitors (4-chlororesorcino and diethyldithlocarbamate), and two reducing agents (ascorbic acid and cysteine) were added to the incubation solution. In other experiments the inhibitors and reducing agents were used to pretreat the mung bean cuttings prior to incubation with catechol or catechol and IAA. In either case the activity of catechol or catechol plus IAA was not reduced.

The question of the mode of action of catechol remains to be answered as does the identity of cofactor 4. Although the R_f's of catechol and cofactor 4 are similar, the ultraviolet absorption spectrum of catechol is entirely different from the end absorption obtained with the cofactor. Solubility characteristics are also different. However, preliminary information from infrared analysis indicates that hydroxyl groups are present in the structure of cofactor 4.

Summary

A procedure for purifying the rooting cofactors obtained from Hedera and Hibiscus is described. The chemical and physical data obtained to date suggest that cofactor 4 is a phenolic compound. It is shown that in order for a phenolic compound to be biologically active in the mung bean rooting test, an ortho-dihydroxy structure with an open para position is required. Although indirect evidence suggests that the first step in the stimulation of rooting by catechol is oxidative, phenolase inhibitors and reducing agents have not reduced the biological activity of catechol.

TABLE 1

SOLUBILITY CHARACTERISTICS OF
COFACTOR 4 AT ROOM TEMPERATURE

Heptane	Insoluble
Petroleum ether	Insoluble
Carbon tetrachloride	Soluble
Benzene	Soluble
Chloroform	Soluble
Ether	Soluble
Acetone	Soluble
Ethanol	Soluble
Methanol	Soluble
Water	Very slightly soluble

TABLE 2

REACTION OF COFACTOR 4 TO SEVERAL REAGENTS

Reagents*	Result
Diazotized Sulfanilic Acid	Red Color
Coupling with B-anphthol	Pink Color
Sucrose in HCl and Ethanol	Red Color
$FeCl_3$ Solution	Slight grey color
Nitrobenzenediazonium Fluoroborate	Brown changing to red

*From Block, R. J. et al. 1958. A Manual of Paper Chromatography and Paper Electrophoresis Academic Press, Inc., New York. 710 p.

TABLE 3

REACTION OF CATECHOL
WITH INDOLEACETIC ACID (IAA)

Treatment	Ave.No. of Roots Per Cutting
H_2O	7.3
IAA 5 X 10^{-6}M	16.0
Catechol 2 X 10^{-4}	27.3
Catechol + IAA	55.1

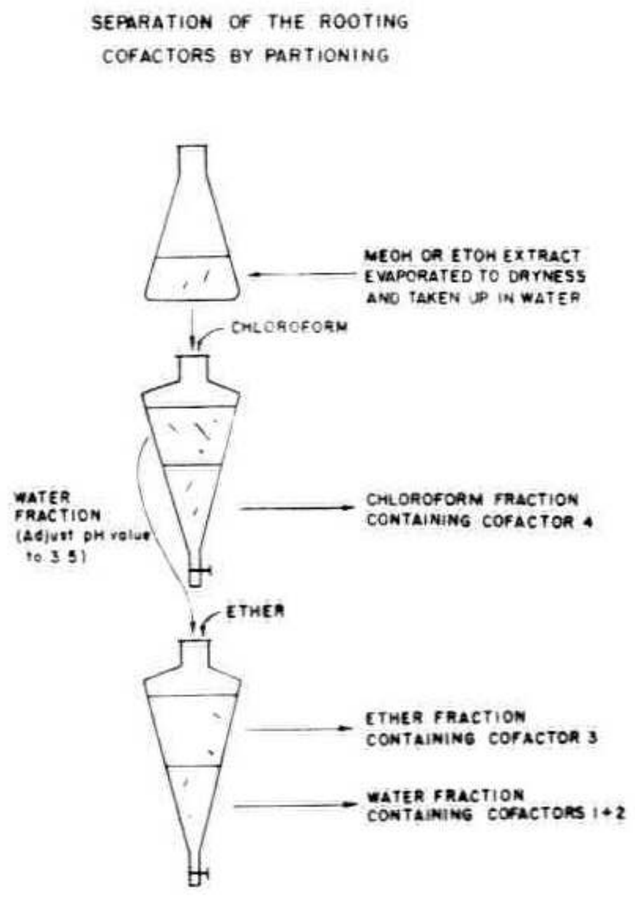

Figure 1

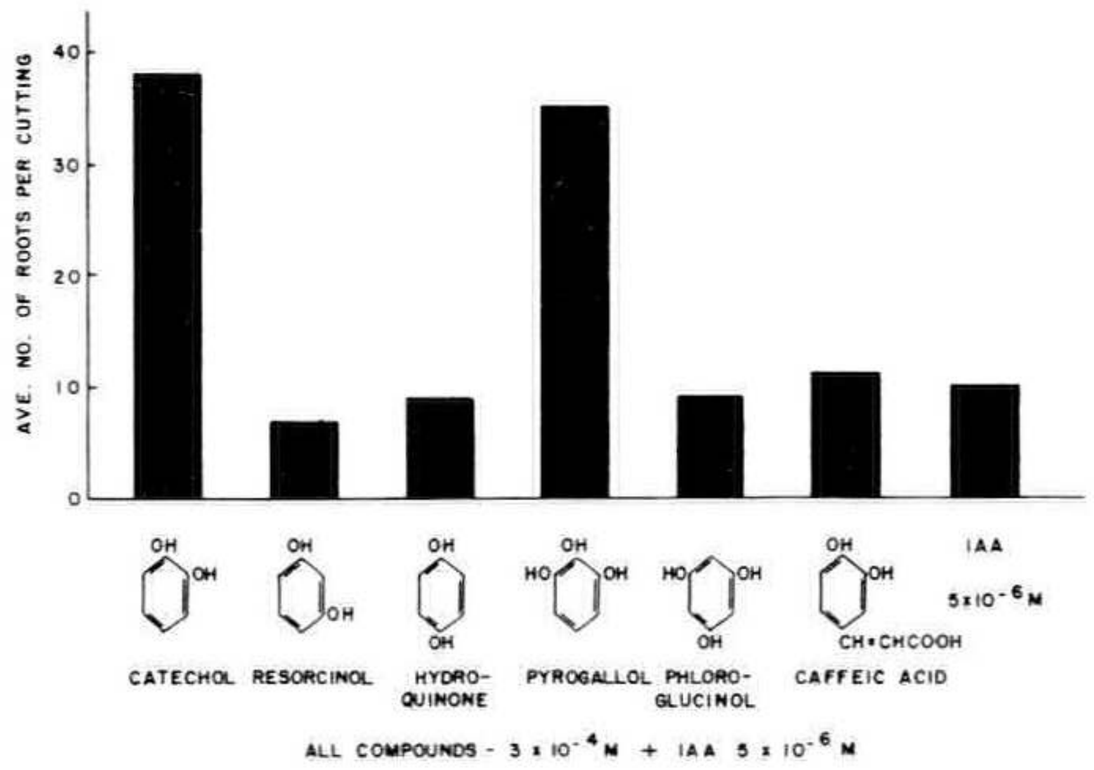

Figure 3
The effect of the position of hydroxyl group substitution upon root initiation in mung bean cuttings.

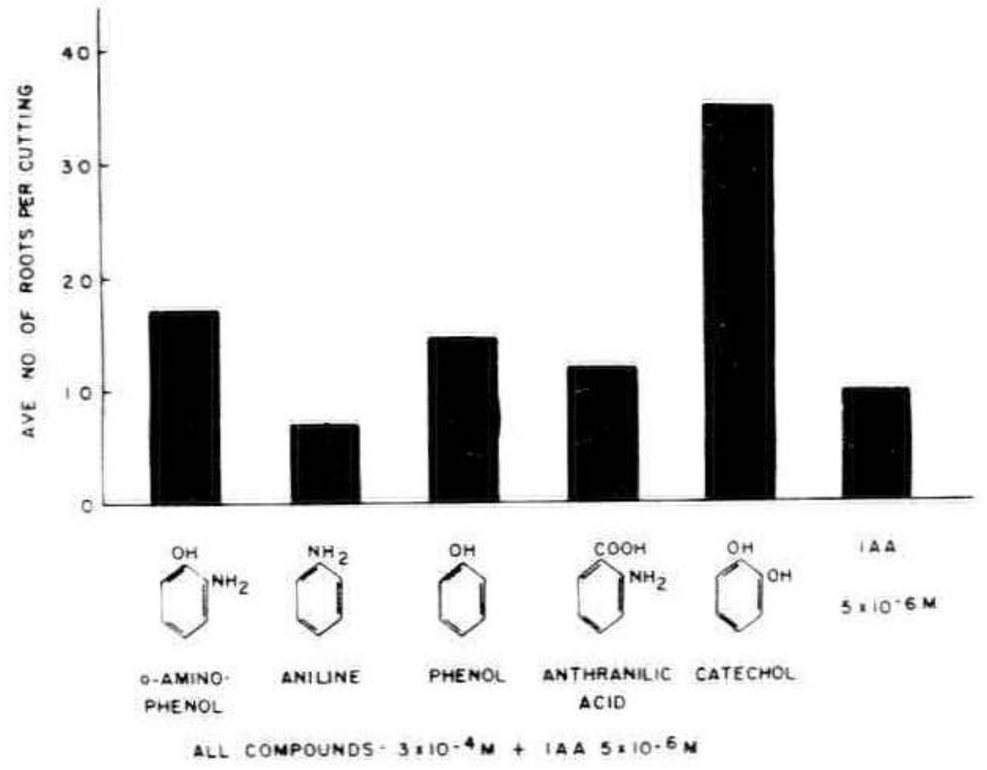

Figure 2
The effect of amino and hydroxyl group substitution upon root initiation in mung bean cuttings.

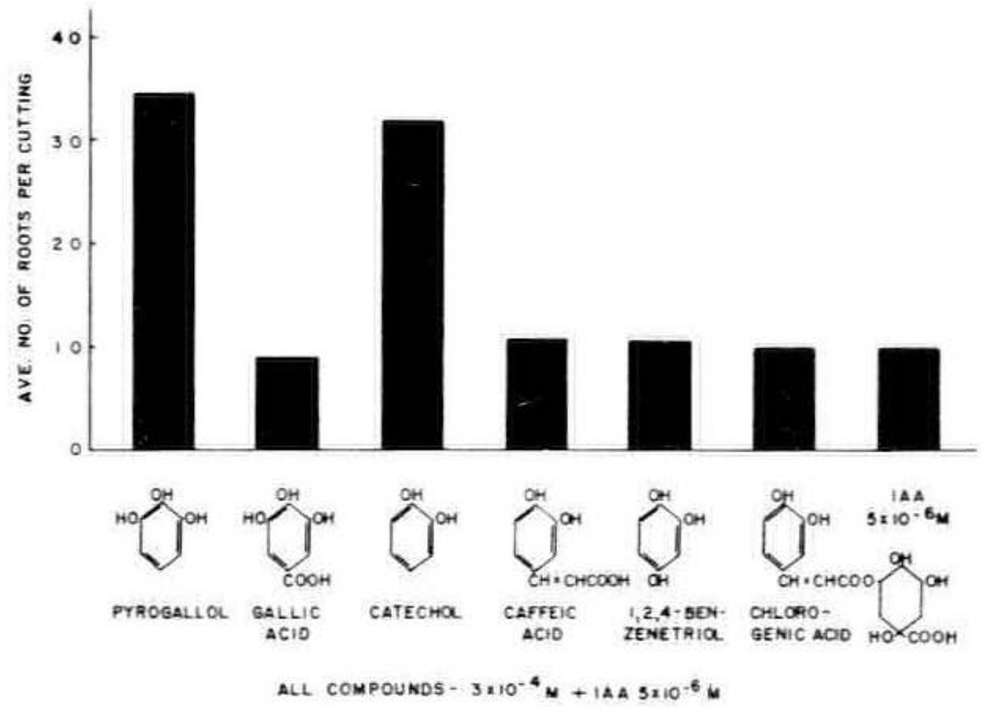

Figure 4
The effect of para substitution upon root initiation in mung bean cuttings.

Literature Cited

1. Bouillenne, R. and M. Bouillenne-Wairand. 1955. Auxines et Bouturage. Report 14th Internat. Hort. Congress, Vol 1: 231-238.

2. Hess, C. E. 1959. A Study of Plant Growth Substances in Easy and Difficult-to-Root Cuttings. Proceedings Plant Propagators Society, Vol 9:39-45.

3. Leopold, A. C. and T. H. Plummer. 1961. Auxin-Phenol Complexes. Plant Physiology 36:589-592.

PRESIDENT VAN HOF: Are there any questions?

MR. ROBERT HARE (Long Beach, Miss.): Have you tried the fourth cofactor itself?

DR. HESS: Most of the work so far has been on using the mung bean, although we have had some experiments with it on chrysanthemum.

If a plant is very difficult-to-root it will lack probably all of the cofactors, and if it is intermediate in rooting ability, it may lack two or three out of the four. When we reapply the cofactor, whether we get a response or not is dependent on whether that is the cofactor which is missing. In other words, we feel that we won't really have the complete answer until we have all of the cofactors identified and reapply all of them simultaneously.

MR. RONALD DeWILDE, JR.: Do you feel other species would have additional factors?

DR. HESS: We have checked Hibiscus, chrysanthemum and Hedera, and we find in all these tissues the four cofactors. We have been working with the fourth cofactor primarily from two plants. Hedera and chrysanthemum turn out to have a large amount of the fourth cofactor. We find there is a slight difference in the two. Whether this will affect the promotive ability of the cofactor, we don't know as yet.

MR. ROBERT HARE: Apparently the mung bean is deficient in all four cofactors.

DR. HESS: That is true. It has a small amount of cofactors present. Some have to be present in order to get expression of a single cofactor.

PRESIDENT VAN HOF: Next on our program is Wyatt Osborne, who is going to tell us all about soil sterilization.

DR. WYATT W. OSBORNE: Thank you, Mr. Chairman, Ladies and Gentlemen: It is a pleasure to be here and I listened with great interest to Dr. Hess' presentation on the cofactors that influence the

rooting of plants. My work, I think, is vital in the rooting of plants. I couldn't help but reflect upon it at the time he made his presentation. My work at VPI is in the field of plant pathology and nematology, that particular field of proper protection from various plant diseases.

Dr. Osborne presented the following paper.

SOIL STERILIZATION AND FUMIGATION

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Plant diseases become a limiting factor where plant propagation areas are used intensively. There are four general groups of causal agents of plant diseases; fungi, bacteria, nematodes, and viruses. Following are some pertinent characteristics of each of these disease producing organisms. Fungi - generally microscopic, filamentous organisms that reproduce by spores (seeds), commonly air borne. Spores produce germ tubes that may penetrate directly into the plant tissue or through natural openings or wounds, and cause infection. Bacteria - microscopic, one-celled organisms which cannot withstand desiccation. None of the plant disease bacteria produce spores, thus they cannot remain alive while being carried great distances in the air as can fungi. Bacteria enter plants through natural openings or wounds. Nematodes - Eel-shaped, microscopic organisms that may inhabit the soil in immense numbers. They require water for movement. Most frequently injury is caused by feeding in or on the roots but some can infect the upper part of the plant. Nematodes reproduce by eggs. They are not commonly transported by wind as are fungus spores. Viruses - Submicroscopic entities which must be transported from plant to plant by man's activities or by insects or mites. Viruses cannot penetrate directly but must be placed into a wound or injected during insect feeding. So far as is known, viruses cannot multiply outside their host plants or insect vectors and in some cases become non-infectious when the host plant dies.

Many disease causing organisms referred to as soil inhabitants are capable of residing in the soil for a period of several years, without access to its host. This is especially true of certain fungi; notable examples being Rhizoctonia the principal cause of seedling camping-off and stem canker, Fusarium and Verticillum which cause wilts and Pythium which causes damping-off and root-rot of seedlings and cuttings of susceptible plants. Other organisms, called soil invaders, do not persist as long in the soil. Many of them survive only as long as the host material, either living or dead, persists as a substrate for their existence. When these disease organisms are not eliminated from the soil plant growing, at best, is an inefficient operation that does not realize the maximum profit potential.